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(54) Title: METHOD OF DETECTING CANCER INVOLVING FRAGMENTS OF CYTOKERATIN 18 AND CORRESPONDING ANTIBODIES

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
Gln Met Glu Gln Leu Asn Gly Ile Leu Leu His Leu Glu (1)

Gln Lys Thr Thr Thr Arg Arg Ile Val Asp Gly Lys Val Val Ser Glu
Thr Asn Asp Thr Lys Val Leu Arg His (2)

(57) Abstract

A method of detecting cancer in a patient, in which the amount of protein fragments comprising at least one amino-acid sequence selected from the group consisting of the amino-acid sequence (SEQ ID NO: 1) and homologues thereof, and the amino-acid sequence (SEQ ID NO: 2) and homologues thereof, is measured in a sample of body fluid or suspected cells from said patient, is disclosed. Also, the above polypeptides, fusion proteins, RNA and DNA sequences relevant to the polypeptides and fusion proteins, antibodies raised against the polypeptides, fusion proteins, corresponding antigen-mimicking structures or synthetic antibody-mimicking structures complementary to the pertinent epitopes, and diagnostic kits, are disclosed.

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Method of detecting cancer involving fragments of cytokeratin 18 and corresponding antibodies.

The present invention relates to a method of detecting cancer in a patient based on detection of certain protein fragments in a sample of body fluid from said patient. Further, the invention relates to two polypeptides and their homologues, to fusion proteins comprising such polypeptides, to RNA or DNA sequences coding for the amino-acid sequence of such polypeptides, to antibodies raised against such polypeptides or fusion proteins, and to diagnostic test kits.

Background

It is of utmost importance to be able to diagnose cancer diseases and to predict patient survival with or without therapy. Monitoring of the efficacy of treatment with chemotherapeutics, hormones, radiotherapy etc. is important for doctor decisions. The aim of therapeutic treatment of cancer is to inhibit or eliminate uncontrolled cell growth. Failure or success of such treatment implies progressive cancer or no growth of tumors.

There are several soluble serum and other body fluid markers that have been used as biological markers in the detection and the follow-up of patients with cancer, such as hormones, oncofetal antigens, enzymes, growth factors, placental proteins, glycoproteins, mucins and others.

Tissue polypeptide antigen (TPA) was first isolated from various types of human cancers by Björklund B. and Björklund V. in 1957 (Antigenicity of pooled human malignant and normal tissue by cyto-immunological technique: Presence of an insoluble heatlabile tumor antigen. Int Arch Allergy 1957; 10: 153-184). TPA is produced by both normal and malignant cells. Elevations in serum are believed to be

related to cell turnover. Monoclonal antibodies directed to TPA are useful in the monitoring of patients with various malignancies.

Measurements of TPA have been used in a number of human malignant tumours for follow-up of diagnosed patients, control of treatment and for the detection of recurrence. The clinical utility of measured TPA has been reported for breast cancer (Barak, M. et al, CA-15.3, TPA and MCA as markers for breast cancer. Eur J Cancer 1990; 26: 577-580; Nemoto, T. et al, Human tissue polypeptide antigen in breast cancer. J Natl Cancer Instit 1979; 63: 1247-1253), lung cancer (Buccheri, G.F. et al, Clinical value of multiple biomarker assay in patients with bronchogenic carcinoma. Cancer 1986; 57: 2389-2396; Buccheri, G.F. et al, Usefulness of tissue polypeptide antigen in staging, monitoring and prognosis of lung cancer. Chest 1988; 93: 565-570), bladder cancer (Adolphs, H.D. et al, Significance of plasma tissue polypeptide antigen determination for diagnosis and follow-up of epithelial bladder cancer. Urol Res 1984; 12: 125-128; Carbin, B.E. et al, Urine - TPA (tissue polypeptide antigen), flow cytometry and cytology as markers for tumours invasivness in urinary bladder carcinoma. Urol Res 1989; 17: 269-272), prostate cancer (Lewenhaupt, A. et al, Tissue polypeptide antigen (TPA) as a prognostic aid in human prostatic carcinoma. Prostate 1985; 6: 285-291) and ovarian carcinoma (Crombach, G. et al, Serum levels of TPA in patients with gynaecological cancer. Protides Biol Fluids 1984; 31: 441-444; Panza, N. et al, Cancer antigen 125, tissue polypeptide antigen, carcinoembryonic antigen and β-chain human chorionic gonadotropin as serum markers of epithelial ovarian carcinoma. Cancer 1988; 61: 76-83).

Björklund et al published in 1987 monoclonal antibody mapping of TPA, which gave 35 different epitopes, (Biochemische und morphologische Grundlagen von TPA: Fortschritte in Richtung auf einem allegemeinen Marker fur aktive Tumoren durch monoclonale kartierung. In: Luthgens M, Schlegel G, eds. Tumormarkersystem CEA-TPA. TumorDiagnostik Verlag, 1987; 14-30). Two of these epitopes were identified to be related to human carcinoma. As a result of this monoclonal mapping of epitopes monoclonal antibodies (MAbs) were raised against these epitope structures by the application of hybridoma technology according to Kühler and Milstein.

Monoclonal antibodies yield a highly reproducible and replenishing source of antibodies compared with a limited supply of polyclonal antibodies obtained from immunized animals. Monoclonal antibodies also provide a more specific reagent as monoclonals may react with only one specific antigenic determinant present in a given antigen molecule, whereas conventional polyclonal antisera contain a mixture of antibodies reacting with different determinants present in a single antigenic molecule.

Immunoassays were developed based on one specific monoclonal antibody in combination with polyclonal horse antibody. Such immunoassays have been marketed by Beki Diagnostics AB, Sweden, under the tradename "TPS" as tumor marker for measuring TPA in serum and other body fluids. The assay measures tumor activity as opposed to such tumor marker assays which measure tumor mass. However, the amino-acid sequence of the protein fragment to which the monoclonal antibody used in the TPS assay specifically binds, has hitherto not been known.

The present invention is based on the finding of two distinct amino-acid sequences on protein fragments in body fluids, to one of which the monoclonal antibody of "TPS" binds and to the other of which another of the monoclonal antibodies published in 1987 binds, respectively. Said protein fractions are, as is evident from the above cited earlier work, indicative of tumor cell activity.

The above two amino-acid sequences form the bases of the different aspects of the invention. In a data base search it was revealed that the amino-acid sequences of the two polypeptides of the invention correspond to the amino acids 318 to 346 and 406 to 430, respectively, of human cytokeratin 18 (CK 18) [The amino-acid numbering starts with the initial methionine]. The complete amino-acid sequence of cytokeratin 18 has been published (Oshima, R.G., Millan, J.L., and Ceccena, G., Comparison of mouse and human keratin 18: a component of intermediate filaments expressed prior to inplantation. Differentiation 33 (1986) 61-68).

Description of the inventi n

One aspect of the invention is directed to a method of detecting cancer in a patient, in which the amount of protein fragments comprising at least one amino-acid sequence selected from the group consisting of the amino-acid sequence (SEQ ID NO: 1)

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

and homologues thereof, and

the amino-acid sequence (SEQ ID NO: 2)

Gln Lys Thr Thr Thr Arg Arg Ile Val Asp Gly Lys Val Val Ser Glu
1 5 10 15

Thr Asn Asp Thr Lys Val Leu Arg His
20 25

and homologues thereof

is measured in a sample of body fluid or suspected cells from said patient, and the amount obtained is compared with a reference, whereby elevated levels indicate cancerous activity in said patient.

Elevated levels may also be obtained in case the patient in question suffers from liver or kidney damage, or is subject to proliferative activity in connection with repair processes e.g. after pneumonia. However, such other causes for elevated levels are easily identified by the patient´s doctor.

In this context a homologue of said amino-acid sequence is a homologous sequence having some amino-acid substitutions, extensions and/or deletions which do not lead to the elimination of the capability of the homologous sequence to bind to the same antibodies as the amino-acid sequence SEQ ID NO: 1

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

and/or to bind to the same antibodies as the amino-acid sequence SEQ ID NO: 2

Gln Lys Thr Thr Thr Arg Arg Ile Val Asp Gly Lys Val Val Ser Glu
1 5 10 15

Thr Asn Asp Thr Lys Val Leu Arg His
20 25

Further, a homologue of said amino-acid sequences is any sequence which is sufficiently homologous at the nucleotide level to be recognized by a RNA or DNA sequence complementary to a RNA or DNA sequence which codes for said amino-acid sequences.

Examples of homologues of the amino-acid sequence SEQ ID NO: 1 are the amino-acid sequence SEQ ID NO: 3

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile
20

and the amino-acid sequence SEQ ID NO: 4

Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu Gln Met Glu Gln
1 5 10 15

Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

Homologous sequences, in addition to the amino-acid sequences SEQ ID NO:3 and SEQ ID NO: 4, which with the aid of ELISA absorbance studies have been found to bind to the same antibody as the amino-acid sequence SEQ ID NO: 1 are amino-acid sequences selected from the group consisting of the amino-acid sequence SEQ ID NO:5

Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu Gln Met Glu Gln Leu Asn
1 5 10 15

Gly Ile Leu Leu His, and corresponding amino-acid sequences
20

wherein Leu in position 1 is substituted by Lys, Gly, Ala or Glu,
Arg in position 2 is substituted by Lys, Gly, Ala or Glu,
Glu in position 3 is substituted by Lys, Gly, Ala or Tyr,
Val in position 4 is substituted by Lys, Gly, Ala or Glu,
Glu in position 5 is substituted by Lys, Gly, Ala or Tyr,
Ala in position 6 is substituted by Lys, Gly, Tyr or Glu,
Arg in position 7 is substituted by Lys, Gly, Ala or Glu,
Tyr in position 8 is substituted by Lys, Gly, Ala or Glu,
Ala in position 9 is substituted by Lys, Gly, Tyr or Glu,
Gln in position 11 is substituted by Lys, Ala or Glu,
Ala in position 12 is substituted by Lys, Ala or Glu,
Asn in position 16 is substituted by Lys, Gly, Ala or Glu,

Gly in position 17 is substituted by Tyr, Ala or Glu,
Leu in position 19 is substituted by Lys, Gly, Ala or Glu,
Leu in position 20 is substituted by Lys, Gly, Ala or Glu,
and Glu in position 21 is substituted by Lys, Gly, Ala or Glu.

Preferred homologues are those
in which Arg in position 2 is substituted by Ala or Glu,
Val in position 4 is substituted by Ala or Glu,
Ala in position 6 is substituted by Glu,
Arg in position 7 is substituted by Lys or Ala,
Ala in position 9 is substituted by Glu,
Gln in position 11 is substituted by Ala or Glu,
Asn in position 16 is substituted by Lys or Ala,
Leu in position 20 is substituted by Lys or Ala,
and His in position 21 is substituted by Gly or Ala.

The reference to be used in the comparison of this aspect of the invention is e.g. a correspondingly obtained amount in a sample of body fluid or relevant cells from healthy patients or cancer patients with no evidence of disease (NED).

The method of the invention is especially useful for detecting carcinoma activity, and to a certain extent also other types of malignant tumours.

Any technique which can quantify the amount of polypeptide fragments comprising at least one of the amino acid sequences SEQ ID NO: 1, 2 and homologues thereof in a sample of body fluid such as blood, plasma, serum, urine, ascites, cerebrospinal fluid, pleura fluid, cyst fluid or suspected cells, can be used in the method of the invention. Monoclonal antibodies which specifically bind to said fragments can be used in different immunoassays, optionally labelled in accordance with the actual assay used.

In one embodiment of the method of detecting cancer according to the invention the measurement is performed with the aid of an immunoassay. Also, detection systems

adapted for the use of antibody-site carrying structures and/or antibodies may be used. Specific examples of the numerous immunoassays which may be used in the invention are Enzyme-linked immunosorbent assay (ELISA), Radioimmunoassay (RIA, IRMA), Fluorescence immunoassay (FIA), Luminiscence immunoassay (LIA), Dissociation enhancement time-resolved fluoroimmunoassay (DELFIA).

Another aspect of the invention is directed to a polypeptide selected from the group consisting of

a polypeptide having the amino-acid sequence (SEQ ID NO: 1)

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

and homologues thereof, and

the amino-acid sequence (SEQ ID NO: 2)

Gln Lys Thr Thr Thr Arg Arg Ile Val Asp Gly Lys Val Val Ser Glu
1 5 10 15

Thr Asn Asp Thr Lys Val Leu Arg His
20 25

and homologues thereof.

A further aspect of the invention is directed to a fusion protein comprising a polypeptide of the invention.

A polypeptide or fusion protein according to the invention can be used i.a. as hapten or antigen to raise antibodies against such a polypeptide or fusion protein, respectively, especially monoclonal antibodies.

Thus, yet another aspect of the invention is directed to an antibody raised against a polypeptide of the invention or fusion protein of the invention, corresponding synthetic antigen-mimicking structures or synthetic antibody-mimicking structures complementary to the pertinent epitopes.

Still another aspect of the invention is directed to a RNA or DNA sequence coding for the amino-acid sequence of a polypeptide according to the invention or a fusion protein according to the invention. Such RNA and DNA sequences are i.a. useful in the production of the polypeptides and fusion proteins of the invention.

A different aspect of the invention is directed to a RNA or DNA sequence complementary to a RNA or DNA sequence coding for an amino-acid sequence of a polypeptide according to the invention. These complementary RNA and DNA sequences may be used to locate the RNA or DNA sequences which code for the protein fractions comprising the amino-acid sequence which is to be detected in the method of detecting cancerous activity according to the invention.

An additional aspect of the invention is directed to a diagnostic test kit comprising, as a diagnostic antibody, an optionally labelled antibody according to the invention, or as a gene detector, an optionally labelled RNA or DNA sequence according to the invention. Examples of such optional labels are a radioactive isotope, a lanthanide marker, a fluorescent marker, a luminescent marker or an enzyme marker.

Optionally all the reagents in the diagnostic test kits of the invention are contained in separate sealed test tubes or vials marked with specific labels, and are accompanied by instructions for use.

Synth sis of the polypeptides of the invention

The polypeptides of the invention can be produced by any known method of producing an amino-acid sequence, such as, controlled degradation of a purified protein by proteases or other chemical methods (Allen G., Sequencing of proteins and peptides, 1989, Elsevier Science Publishers B.V.). Chemical synthesis is commonly performed by coupling of the amino acid residues or peptide fragments to one another in correct order in liquid phase to produce the desired peptide. Another common strategy is the coupling of the amino acids to one another starting with a solid phase (resin) to which the C-terminal of the first amino acid is coupled, whereupon the C-terminal of the next amino acid is coupled to the N-terminal of the first amino acid, etc, finally releasing the built-up peptide from the solid phase (so called solid-phase technique).

The polypeptides of the invention were synthesized by such solid-phase technique (reviewed in Atherton E. and Sheppard R C 1989. Solid Phase Peptide Synthesis a practical approach. IRL Press at Oxford University Press ISBN 019-963066-6), and they were purified by reverse-phase high performance liquid chromatography (HPLC). The amino-acid sequences were checked by ion spray mass spectrometry (Van Dorsselear et al, 1990, Application of electrospray mass spectrometry to characterisation of recombinant proteins up to 44kDa. Biomed. Environ. Mass Spectrom. 19, 692-704).

Construction and production of fusion proteins of the invention

800,000 phages of the human prostate cDNA library (λgtII cDNA bank from Clontech) were screened with TPS antibody and 4 positive phages were identified which were subcloned until a homogeneous population was obtained (Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
The bacterial expression vector pET3c (Studier et al, 1990, Methods of Enzymology 185, 60-89) was used to produce fusion proteins by cloning Bg1II/BamHI fragments

of CK 18 cDNA and PCR engineered deletions thereof in phase into the BamHI site of pET3a. The fusion proteins consisting of 260 amino acids of the T7 gene 10 protein and different parts of CK 18 were expressed. The syntheses of the fusion proteins were induced by isopropyl-ß-D-thiogalactopyranoside (IPTG) and the fusion proteins were purified from sonicated cells by preparative SDS electrophoresis (Hager, D.A. and Burgess, R.R. 1980, Anal.Biochem. 190, 76)

Example 1

The availability of two monoclonal antibodies each recognizing a different epitope on the same antigen, namely antibodies binding to the SEQ ID NO: 1 and 2, i.e. MAb1 and MAb2, respectively, permits the application of dual monoclonal immuno-radiometric assay. One of the monoclonal antibodies (MAb1) is coated on a solid phase, polystyren beads, with enhanced surface by incubation with 14 µg MAb1/ml in 0.1 M bicarbonate buffer, pH 9.6, for 24 hours at 4°C. The beads are washed twice with PBS (0.014 M Na-K phosphate, pH 7.5, containing 0.15 M NaCl) and remaining sites are blocked with PBS containing 1% bovine serum albumin and 0.1% Tween 20. This MAb1 antibody will capture antigen when body fluids containing protein fragments comprising the epitope structure are added to the solid-phase bound antibody by binding to one of the epitope structures. The second monoclonal antibody (MAb2) is labelled with radioisotope (125-I) by the chloramine-T method (Greenwood F.C., W.M. Hunter, and j.S. Glover. 1963 The preparation of 131-I labelled human growth hormone of high specific radioactivity. Biochem. J. 89: 114-123) and purified by size exclusion chromatography on a Superdex column. MAb2 binds to the antigen by reacting with a different epitope structure, well separated from the MAb1 binding region.

Serum from blood donors or cancer patients are tested by mixing 0.100 ml of each sample with 0.100 ml of 125-I labelled MAb2, and adding a bead-coupled antibody (MAb1). After incubation during agitation for 2 hours the solid phase is washed with water to wash away unbound antigen and antibody, and the amount of bound radioactivity is determined in a gamma counter.

Assay of protein fragments in body fluids

In order to measure the amount of protein fragments comprising both of the amino-acid sequences SEQ ID NO:1 and 2 in patient sera, the described dual monoclonal assay was applied. A serum sample from the patient is added to the reaction well, the second antibody (MAb2) labelled with isotope is also added followed by mixing. The first antibody (MAb1), the catching antibody bound to solid phase, is added to each well and subsequently the reaction mixture is incubated for a specified time period, washed and measured in a gamma counter.

Positive samples from the patient are defined as a marker level exceeding the reference limit of 80 U/L. This cut-off value is based upon a study of 195 blood donors where the upper limit was 80 U/L (95% percentile).

Table 1 illustrates the results of Example 1. In the Table the results of blood donors, patients with benign disorders and malignant diseases in various stages of development (primary diagnosis) are presented. It should be noted that elevated levels are seen in certain benign diseases in connection with increased proliferation activity due to healing of inflammatory damage, increased proliferation in connection with beningn hyperplasia (prostate hyperplasia), or simply due to decreased excretion from the liver or the kidneys , which normally remove the antigen (proliferation product) from the serum.

Table 1

	Total number	Positive
Blood donors	90	0
Benign disorders		
Prostatic hyperplasia (BPH)	24	2
Renal insufficience	23	15
Liver cirrhosis	21	19
Chronic hepatitis	13	11
Pneumonia	16	4
Chronic bronchitis	17	3
Malignant disease		
Breast	129	116
Ovary	40	27
Cervix	32	25
Lung	70	49
Prostate	64	22
Bladder	56	22
Kidney	16	11
Gastrointestinal	40	37
Head and neck	22	11

Monitoring of a cancer patient

A dual monoclonal assay was repeatedly performed during hormone treatment of a breast cancer patient with Tamoxifen. Table 2 demonstrates the monitoring of said patient with the assay . The clinical diagnosis of the patient prior to treatment was distant bone metastases and malignant pleural effusion.

The clinical assessment indicated progressive disease at the initiation of the hormonal treatment, and after 3 months the clinical finding was stable disease. At that time the signal had decreased significantly from an elevated value at the start of the treatment.

At month 7, the clinical assessment was progressive disease, and at that occation the marker level was markedly elevated. Already 3 months prior to the clinical finding of recurrence this was indicated by the rise in the signal (lead-time).

Table 2

Months	Signal (U/L)♦	Clinical assessment
0	475	Progressive disease
0.5	210	
0.8	205	
3.0	70	Stable disease
4.2	110	
5.0	250	
7.0	270	Progressive disease

♦ Normal range of marker level < 80 U/L .

Example 2

Design of an alternative test system

Instead of using two monoclonals in the serodiagnostic test, a system was designed where MAb1 on the solid face was substituted with horse antibodies to cancer cells (HeLa). Otherwise the test was performed as described in Example 1.

Out of 30 cancer patients with various gastrointestinal malignant diseases, 28 were positive when the same critera as in Example 1 were applied.

Example 3

In vivo applications of antibodies directed against the sequence ID NO: 1 or 2

Labelling of antibodies directed against sequence ID NO: 1 or 2 with radioactive isotopes like 131-I, 123-I, 111-In or 99m-Tc, and injection of these antibodies intravenously to cancer patients, make it possible to detect tumors in the body by scintigraphy without background subtraction.

In an initial in vivo study 123-I MAb1 (300 Mbq/1.5 mg MAb) was able to localize bone and lung metastases in patients suffering from breast, renal or prostatic cancer.

Example 4

Production of monoclonal antibody against polypeptides SEQ. ID. NO: 1 and 2

As immunogen for production of monoclonal antibodies any type of antigen, including cancer cells (HeLa), which expose SEQ. ID. NO. 1 and/or 2 can be used provided a suitable selection system is available. Immunization with purified cytoskeleton (Achstätter et al 1986, Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. Methods Enzymol. 134 355-371), peptide-carrier protein complex (Antibodies: A laboratory manual ed: Harlow and Lane, 1988 - Cold Spring Harbor Laboratory p 77-87) or purified CK 18 fragment from cancer cells in tissue culture can be done according to methods described in (Antibodies: A laboratory manual ed: Harlow and Lane, 1988 - Cold Spring Harbor Laboratory). Balb/c mouse was injected with fusion proteins consisting of 260 amino acids of the T7 gene 10 protein and amino acids 140-430 of human CK 18. After 3-4 injections the mouse serum was tested for antibodies against human cytokeratin 18. When positive response was obtained, spleen cells were fused with a mouse myeloma cell line. Positive hybridomas were subcloned and then single-cell cloned, after which stable clones were established.

The specificity of the produced antibody was then tested by immunoblotting against 8 fusion proteins with different deletions of the above described fusion protein.

Table 3 Fusion proteins and reactivity of MAbs

Lab. sample	CK 18 aas	Antibody Binding				
		MAb1	MAb2	MAb3	MAb4	MAb5
298	140-298	-	-	+	-	+
323	140-323	-	-	+	-	+
341	140-341	+	-	+	-	+
352	140-352	+	-	+	-	+
372	140-372	+	-	+	-	+
389	140-389	+	-	+	-	+
415	140-415	+	-	+	+	+
430	140-430	+	+	+	+	+

Conclusion regarding epitope localization:

	MAb1	MAb2	(MAb3)	(MAb4)	(MAb5)
Epitope region	318-346	406-430	140-298	381-415	140-298

Example 5

The five monoclonals were tested against known cancer patient sera and blood donors by a two step immunoradiometric assay where the samples were incubated 2 hours with plastic beads coated with horse antibodies to cancer cells (HeLa) and the different MAbs at a concentration of 100 ng/ml. The beads were then washed to remove unbound material, and incubated with 125-I labelled horse anti-mouse IgG for 1.5 hours. After washing unreacted radioactivity off the beads, the radioactivity bound to the beads was measured using a gamma counter.

The results are given in Table 4

Table 4

SAMPLE	MAb1	MAb2	(MAb3)	(MAb4)	(MAb5)
Blood donor (Blg) cpm	2955	4430	744	411	4115
Cancer patients (CaPts) cpm	12990	19942	2814	1332	8182
CaPts/Blg ratio	4.4	4.5	3.8	3.2	2.0

Only hybridomas which produced antibodies which gave high signal for cancer patients resp low signal for blood donors were selected, i.e. in this example MAb1 and MAb2.

Example 6

The antibodies MAb1 and MAb2 which are directed against the sequences ID. NO: 1 and 2, respectively, of the invention were compared with 4 commercially available monoclonal antibodies recognizing cytokeratin 18. These 4 monoclonal antibodies, together with MAb2, did not bind to the sequence ID. NO: 1. According to the experiments demonstrated in Table 5, which were performed in accordance with the assay format described in Exampel 5, the 4 commercially available antibodies are not suitable for the described assay (cf. column "cancer patient" in Table 5). CBL 210 (Cymbus Bioscience Ltd), an anti-cytokeratin 18 monoclonal antibody, is raised in mouse ascitic fluid recognizing adenocarcinomas, undifferentiated carcinomas and carcinomas of cervical and hepatocellular origin. MON 3006 (Sanbio bv-biological products) recognizes cytokeratin 18 (immunoblotting) and do not show crossreactivity with other cytokeratins. Monoclonal anticytokeratin peptide 18, C 1399 (Sigma Chemical Co), is raised against keratin from bovine mammary gland epithelial cell line and reacts specifically with simple epithelia. RGE 53

(EuroDiagnostics) is a monoclonal keratin antibody raised against HeLa cells and recognizing cytokeratin 18 in glandular epithelium.

Cytokeratin 18 antigen, used in these experiments, was isolated from the cytoskeleton of human colon adenocarcinoma cells (WiDr) by following a procedure described by Achstaetter et al, Methods in Enzymology 134 (1984) 355-371 with minor modifications. The isolated cytokeratin fraction was further purified by preparative SDS-PAGE. The protein band corresponding to cytokeratin 18 was extracted from the gel using 0.2% SDS in water for 18 h at room temperature. The protein fraction was precipitated by aceton/water and the precipitate was dissolved in 8 M urea, 0.1 M TRIS-HCl, pH 8.0.

A cytokeratin 18 fragment (14 kDa), from the cell supernatant of human colon adenocarcinoma cell line WiDr was purified using as probe monoclonal MAb1. The 14 kDa component was purified about 30000-fold in a 7% yield by a 5-step procedure; 1) 50% (w/v) ammonium sulfate precipitation of the cell culture medium 2)hydrophobic interaction chromatography on Phenyl Sepharose 3) Sephacryl S-300 gelfiltration chromatography in dissociating medium (8 M urea dissolved in 0.1 M TRIS-HCl, pH 8.0) 4) anion exchange chromatography on Q Sepharose and 5) reverse phase chromatography (acetonitrile gradient). SDS-gradient PAGE of the 14 kDa fraction revealed a pure protein component. The amino acid sequence analysis identified the 14 kDa component as a fragment of human cytokeratin 18 with the N-terminus starting at position 284 of the parent molecule and ended at position 410 of human cytokeratin 18.

Table 5

Mab / Antigen	MAb1	MAb2	CBL210	MON3006	C1399	RGE53
Cytokeratin 18 45 kDa (cpm)	14751	7377	46216	7441	107229	255
Cytokeratin 18 fragment 14 kDa (aa 284-410) (cpm)	90170	212	88	121	253	80
Cancer patient (cpm)	12990	19942	1729	490	848	339
Blood donor (cpm)	2955	4430	309	645	1608	1652

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Beki AB
(B) STREET: Fredsforsstigen 22
(C) CITY: Bromma
(E) COUNTRY: Sweden
(F) POSTAL CODE (ZIP): 161 70
(G) TELEPHONE: 08-290370
(H) TELEFAX: 08-288644

(ii) TITLE OF INVENTION: METHOD OF DETECTING CANCER

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: C-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gln Lys Thr Thr Thr Arg Arg Ile Val Asp Gly Lys Val Val Ser Glu
1 5 10 15

Thr Asn Asp Thr Lys Val Leu Arg His
20 25

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu Gln Met Glu Gln
1 5 10 15

Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 2
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 3
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Tyr

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Tyr

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Tyr or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 8
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Tyr or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:11
(D) OTHER INFORMATION: /note = optionally Lys, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION: /note = optionally Lys, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:16
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:17
(D) OTHER INFORMATION: /note = optionally Tyr, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:19
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:20
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:21
(D) OTHER INFORMATION: /note = optionally Lys, Gly, Ala or Glu

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu Gln Met Glu Gln Leu Asn
1 5 10 15

Gly Ile Leu Leu His
20

CLAIMS

1. A method of detecting cancer in a patient, **characterised** in that the amount of protein fragments comprising at least one amino-acid sequence selected from the group consisting of

the amino-acid sequence (SEQ ID NO: 1)

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

and homologues thereof, and

the amino-acid sequence (SEQ ID NO: 2)

Gln Lys Thr Thr Thr Arg Arg Ile Val Asp Gly Lys Val Val Ser Glu
1 5 10 15

Thr Asn Asp Thr Lys Val Leu Arg His
20 25

and homologues thereof

is measured in a sample of body fluid or suspected cells from said patient, and the amount obtained is compared with a reference, whereby elevated levels indicate cancerous activity in said patient.

2. A method of detecting cancer according to claim 1, wherein said measurement is performed with the aid of an immunoassay.

3. A method of detecting cancer according to claim 2, wherein said immunoassay is selected from Enzyme-linked immunosorbent assay (ELISA), Radioimmunoassay (RIA , IRMA), Fluorescence immunoassay (FIA), Luminiscence immunoassay (LIA), and Dissociation enhancement time-resolved fluoroimmunoassay (DELFIA).

4. A polypeptide selected from the group consisting of

a polypeptide having the amino-acid sequence (SEQ ID NO: 1)

Ala Ser Leu Glu Asn Ser Leu Arg Glu Val Glu Ala Arg Tyr Ala Leu
1 5 10 15

Gln Met Glu Gln Leu Asn Gly Ile Leu Leu His Leu Glu
20 25

and homologues thereof, and

the amino-acid sequence (SEQ ID NO: 2)

Gln Lys Thr Thr Thr Arg Arg Ile Val Asp Gly Lys Val Val Ser Glu
1 5 10 15

Thr Asn Asp Thr Lys Val Leu Arg His
20 25

and homologues thereof.

5. A fusion protein comprising a polypeptide according to claim 4.

6. A RNA or DNA sequence coding for the amino-acid sequence of a polypeptide according to claim 4 or a fusion protein according to claim 5.

7. A RNA or DNA sequence complementary to a RNA or DNA sequence coding for the amino-acid sequence to be detected in the method of claim 1.

8. An antibody raised against a polypeptide according to claim 4 or a fusion protein according to claim 5, corresponding synthetic antigen-mimicking structures or synthetic antibody-mimicking structures complementary to the pertinent epitopes.

9. An antibody according to claim 8, wherein said antibody is a monoclonal antibody or an antigen-binding part thereof.

10. A diagnostic test kit comprising, as a diagnostic antibody, an optionally labelled antibody according to claim 8 or 9, or as a gene detector, an optionally labelled RNA or DNA sequence according to claim 7.

11. A diagnostic test kit according to claim 10, wherein said label is selected from a radioactive isotope, a lanthanide marker, a fluorescent marker, luminescent marker or an enzyme marker.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00532

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/574, G01N 33/68, C07K 14/47, C07K 16/30
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CANCERLIT, EPODOC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 159 CANCERLIT Dialog acc. no. 00920677, A. van Dalen et al: "Facts about TPS, TPA and cytokeratins (Meeting abstract)", Anticancer Res; 12(6A):1829 1992	1-3,8-11
Y		1-11
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X	EP 0337057 A1 (PROGEN BIOTECHNIK GMBH), 18 October 1989 (18.10.89), see Table 5 and 6	1-3,8-11
Y		1-11
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 1995	08 -09- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00532

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4775620 A (ROBERT D. CARDIFF ET AL), 4 October 1988 (04.10.88), column 7, line 10 - line 13	1-3,8-11
Y		1-11
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Y	American Journal of Pathology, Volume 136, No 2, February 1990, H.E. Schaafsma et al, "Distribution of Cytokeratin Polypeptides in Human Transitional Cell Carcinomas, with Special Emphasis on Changing Expression Patterns During Tumor Progression", page 329 - page 343, see Table 1 and "Discussion"	1-11
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Y	The Embo Journal, Volume 1, No 12, 1982, Elke Debus et al, "Monoclonal cytokeratin antibodies that distinguish simple from stratified squamous epithelia: characterization on human tissues" page 1641 - page 1647	1-11
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Y	Cell Tissue Res, Volume 254, 1988, Wilson Savino et al., "Immunohistochemical studies on a human thymic epithelial cell subset defined by the anti-cytokeratin 18 monoclonal antibody" page 225 - page 231	1-11
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Y	Laboratory Investigation, Volume 55, No 4, 1986, Mary Osborn, "Methods in Laboratory Investigation. Differential Diagnosis of Gastrointestinal Carcinomas by Using Monoclonal Antibodies Specific for Individual Keratin Polypeptides" page 497 - page 504	1-11
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A	DE 3923951 A1 (BOEHRINGER MANNHEIM GMBH), 31 January 1991 (31.01.91)	1-11
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INTERNATIONAL SEARCH REPORT

Information on patent family members

31/07/95

International application No.
PCT/SE 95/00532

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0337057	18/10/89	SE-T3- 0337057	
		DE-A- 3815932	03/08/89
		JP-A- 2006756	10/01/90
US-A- 4775620	04/10/88	CA-A- 1336171	04/07/95
		EP-A,B- 0167616	15/01/86
		JP-A- 1047392	21/02/89
		JP-A- 1052800	28/02/89
		JP-B- 6081760	19/10/94
		JP-T- 61501048	22/05/86
		WO-A- 8503132	18/07/85
DE-A1- 3923951	31/01/91	AT-T- 112632	15/10/94
		DE-D- 59007389	00/00/00
		EP-A,B- 0434809	03/07/91
		JP-T- 3502838	27/06/91
		WO-A- 9101499	07/02/91